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DOI: https://doi.org/10.1002/jbm.a.35211

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Patrick Hänseler[a]
Martin Ehrbar[b]
Astrid Kruse[a]
Eliane Fischer[c]
Roger Schibli[c]
Chafik Ghayor[a]
Franz E. Weber[a,d,e]

Running Title: Delivery of rhBMP-2 by Apatite Granules

[a]Oral Biotechnology & Bioengineering, Department of Cranio-Maxillofacial and Oral Surgery, University Hospital Zurich, Zurich, Switzerland and Center for Dental Medicine, University of Zürich, Zurich Switzerland.

[b]Obstetrics, University Hospital Zurich, Zurich, Switzerland

[c]Center for Radiopharmaceutical Sciences ETH-PSI-USZ, Paul Scherrer Institute, 5232 Villigen-PSI, Switzerland

[d]Zurich Center for Integrative Human Physiology, University of Zurich, Zurich, Switzerland

[e]CABMM, Center for Applied Biotechnology and Molecular Medicine, University of Zurich, Zurich, Switzerland

Correspondence:

FE Weber, University Hospital; Dept. of Cranio-Maxillofacial and Oral Surgery, Oral Biotechnology & Bioengineering; Frauenklinikstrasse 24, 8091 Zürich; Switzerland Tel: +41 44 255 5055, Fax: +41 44 255 4179; Email: franz.weber@zzm.uzh.ch

Abstract

Bone morphogenetic proteins (BMPs) are deposited in bone and responsible for osteoinduction. At present there is essentially one product on the market mainly used for spinal fusion purposes where a bovine collagen fleece is applied as delivery system. Now, with the expiration of the patent protection new products utilizing other delivery systems will soon hit the market. The interplay between delivery system and BMP, resulting in a characteristic release profile, is crucial for clinical success. We here report on two apatite based commercially available granules which could potentially be used in a combination product with recombinant human BMP-2 (rhBMP-2). Regardless of their similar chemistry, their interaction with rhBMP-2 differs. Deproteinized bovine bone matrix (DBBM), a clinically well-established bone substitute, has a high affinity to rhBMP-2 and releases only 50% of the growth factor during the first two weeks *in vitro*. Activity of the physio-adsorbed rhBMP-2 is indicated by an enhanced bone augmentation *in vivo*. In contrast, all rhBMP-2 delivered in combination with synthetic hydroxyapatite/β-tricalcium phosphate (HA/TCP) granules is released during the first 24 h. Bone to implant contact is significantly increased for HA/TCP in combination with rhBMP-2 but is still below the level of DBBM alone. For both HA/TCP and DBBM, the released rhBMP-2 is active *in vitro*. Our results suggest that the different release behavior from these two apatite granules is due to the 1000 fold higher specific surface area of DBBM compared to HA/TCP. The sustained release from DBBM suggests that it could be used as a safe delivery system, possibly allowing a reduction of clinical doses of rhBMP-2.

1. Introduction

Bone morphogenetic proteins (BMPs) are growth factors associated with osteoinduction [1]. They recruit and stimulate adult mesenchymal stem cells and preosteoblastic cells to migrate, proliferate and mature into osteoblasts [2, 3]. Currently, recombinant human BMP-2 (rhBMP-2) in combination with a collagen carrier (Infuse®, Medtronic Sofamor Danek, Memphis, Tennessee) is clinically used for lumbar spine fusion and persisting long bone defects. Despite the fact that the application of rhBMP in the clinic is associated with moderate to severe side effects, in particular when used off label as reviewed in [4], the expiration of the patent protection for BMPs will soon yield in new products containing BMPs. One of the side effects, the development of cancer, is linked to a very high rhBMP-2 dosage. The data presented to the US Food and Drug Administration (FDA) on the planned product AMPLIFY™ (Medtronic Sofamor Danek, Memphis, Tennessee) revealed a higher number of cancers in the investigational group compared with the control and led to the disapproval of this product [5]. In essence, this product contained 40 mg of rhBMP-2 on a 20 cc compression-resistant matrix consisting of 15% hydroxyapatite and 85% β-tricalcium phosphate. Lower dosages of 1.5 mg rhBMP-2 per ml graft volume as found in Infuse® (Medtronic Sofamor Danek, Memphis, Tennessee) have not been associated with a higher incidence rate for cancer [5]. Here the major side effect is swelling [6]. Therefore more efficient BMP delivery systems have to be developed where lower amounts of BMP show efficiency.

Bone regeneration is based on osteoconduction, which is the ingrowth of bone into 3-dimensional structures and osteoinduction, which is based on the recruitment and stimulation of mesenchymal stem cells and osteoprogenitor cells to form bone. The transplantation of bone cells is of advantage for the regeneration, if a bone substitute material is applied to facilitate the regeneration [7]. An autologous bone graft contains bone cells per se. Any other source, however, and *in vitro* tissue engineering approaches in particular will certainly increase the costs and efforts substantially and might only be legitimate to be applied in a very limited number of cases. For many clinical cases with small bone defects an osteoconductive bone substitute is sufficient. For larger or even critical sized defects, osteoinductive bone substitute materials may render the application of bone cells redundant. Osteoinductive bone materials can be developed by an optimization of the chemical composition, surface topography, and geometry of the biomaterial, which in turn affect resorption rate and cell-material interactions [8] or by the combination of a bone substitute material with BMPs. Due to the similar chemical composition of natural bone, calcium-phosphate based materials are promising candidates for the development of osteoinductive

bone substitutes of either type. Human bone mineral has been shown to be a promising candidate [9]. Deproteinized bovine bone matrix (DBBM), being the gold standard bone substitute material in dentistry and having a very similar chemistry and structure compared to human bone, would be a clinically well-accepted choice.

Synthetic materials performing equally, however, would be advantageous, since the adjustment range of parameters would be simpler, the production would be possible on larger scales, and there would be no conflicts with religious beliefs of patients regarding implantation of a material derived from another living organism. Many synthetic calcium phosphate based materials are therefore under investigation as bone substitute materials [10, 11] as such or as osteoinductive ceramics [8, 12].

The combination of ceramics with BMPs to render them osteoinductive is a strategy used by nature. It has been shown that rhBMP-2 has a higher affinity to hydroxyapatite compared to other proteins [13]. The mechanisms of protein adsorption to hydroxyapatite are a very complex interplay of various parameters [14-16]. Surface roughness plays a distinct role in protein adsorption to surfaces in general, including hydroxyapatite [17-19]. The specific surface area is certainly another important aspect [20]. For rhBMP-2 in particular, the presence of calcium ions and a slightly acidic pH increase the adsorption of rhBMP-2 from solutions [13, 19].

In a recent study we have investigated the differences in interactions of glycosylated and non-glycosylated rhBMP-2 with DBBM and hydrolysable mPEG hydrogels [21]. Observed strong affinities of both rhBMP-2 variants to DBBM, indicated that DBBM alone might be sufficient as a carrier. This would result in a simpler system and make any decrease of rhBMP-2 activity due to an additional delivery system irrelevant.

We here evaluate clinically applied hydroxyapatite based bone substitute materials as carriers for non-glycosylated rhBMP-2. By radioactive iodine (^{125}I) labeling both rhBMP 2 in solution and adsorbed to hydroxyapatite granules could be detected in parallel in trace amounts using a gamma counter. This allowed the determination of rhBMP-2 release profiles and bioactivity *in vitro*. The relevance of these findings on material growth factor interactions for the *in vivo* situation was then tested in a bone augmentation model in rabbits.

2. Materials and methods

2.1. Detection of the Release Profile

2.1.1 Iodination of rhBMP-2

rhBMP-2 was labeled with radioactive ^{125}I by the Iodogen method. 50 µg Iodogen (Thermo Fisher Scientific, IL, USA) was dissolved in 500 pl ethyl acetate and added to a conical glass tube. The solvent was evaporated gently with a stream of N_2. 297 µl of TU buffer (25 mM Tris, 6 M urea) pH 7.0 containing 100 µg rhBMP-2 were added to the coated glass tube and placed on ice. 3 µl of ^{125}I stock solution (Iodine-125 radionuclide, specific activity ~17 Ci/mg, 10 µM in NaOH pH 8-11, Nr. NEZ033A002MC, Perkin Elmer, MA, USA) were added and the mixture was incubated for 15 min. Free ^{125}I was separated from the labeled protein by loading the reaction mixture on a pre-equilibrated PD-10 column (GE Healthcare, UK) and eluting it with TU pH 7.0 in 1 ml fractions. The radioactivity of the fractions was analyzed by a gamma counter (Cobra II Auto-Gamma, Packard) and the fractions containing the protein were pooled. The protein was concentrated and the buffer was exchanged to 1 mM HCl using centrifugal filter devices (Millipore, MA, USA) with a molecular weight (MW) cutoff of 10 kDa. The iodinated protein was characterized by means of SDS PAGE and FPLC.

2.1.2 SDS PAGE with Autoradiography

Samples containing rhBMP-2 were mixed with 5x sample buffer (10% w/v SDS, 10 mM dithiothreitol (optional, for reduction), 20% v/v glycerol, 0.2 M Tris-HCl pH 6.8, 0.05% w/v bromophenol blue) in a ratio of 1:5. The mixture was incubated at 95 °C for 10 min and, together with MW markers, subjected to gel electrophoresis using a 4% stacking gel and a 15% running gel. The gels were stained with Coomassie (0.01% Coomassie brilliant blue, 50% methanol, 7% acetic acid), destained in a solution containing 25% methanol and 7% acetic acid and finally dried onto filter papers under vacuum at 80 °C. The dried gels were scanned with a standard desktop scanner. If radioactive ^{125}I labels were used, a phosphor screen (Precisely Super Resolution, PerkinElmer) was exposed to the dried gel and a cyclone plus phosphor imaging system (PerkinElmer) was used to digitalize autoradiography intensities. The digital data was edited with OptiQuant 5.0 software (PerkinElmer).

2.1.3 Fast Protein Liquid Chromatography with Autoradiography

Analysis of rhBMP-2 was performed on a Superdex 75 size exclusion column (GE Healthcare, UK) on a fast protein liquid chromatography (FPLC) system from BioRad (CA, USA) connected to a gamma counter as well as a UV detector. Samples were loaded and

eluted using TU buffer pH 7.0 containing 0.5 M NaCl and 0.1% Tween20 at a flow speed of 1 ml/min.

2.1.4 Functionalization of Apatite Granules with rhBMP-2

The here investigated materials were DBBM (kindly provided by Geistlich Biomaterials) and HA/TCP (kindly provided by the Institut Straumann AG, Switzerland) granules, an overview over their properties is given in

. The bone substitute materials were plasma cleaned in a radio-frequency oxygen plasma chamber (RF plasma chamber) at an oxygen pressure of 0.1 mbar to render the surface hydrophilic. In order to adsorb rhBMP-2 or ^{125}I-rhBMP-2 to DBBM or HA/TCP from solution, 21.4 mg of DBBM or HA/TCP were mixed with 50 µl 1 mM HCl containing 1.9 µg rhBMP-2. The mixture was vortexed, briefly spun down (10600xg, 10s) in a centrifuge and incubated overnight. Controls were prepared without the addition of rhBMP-2. Additional DBBM controls were immersed in FBS (Invitrogen, CA, USA) instead of 1 mM HCl. The next day the liquid remaining from the adsorption procedure was transferred to another tube and the concentration of ^{125}I-rhBMP-2 adsorbed to the apatite granules and remaining in solution was determined by a gamma counter.

2.1.5 Release Experiment

The study design was equal to our previous study on interactions of rhBMP-2 with DBBM [21] (

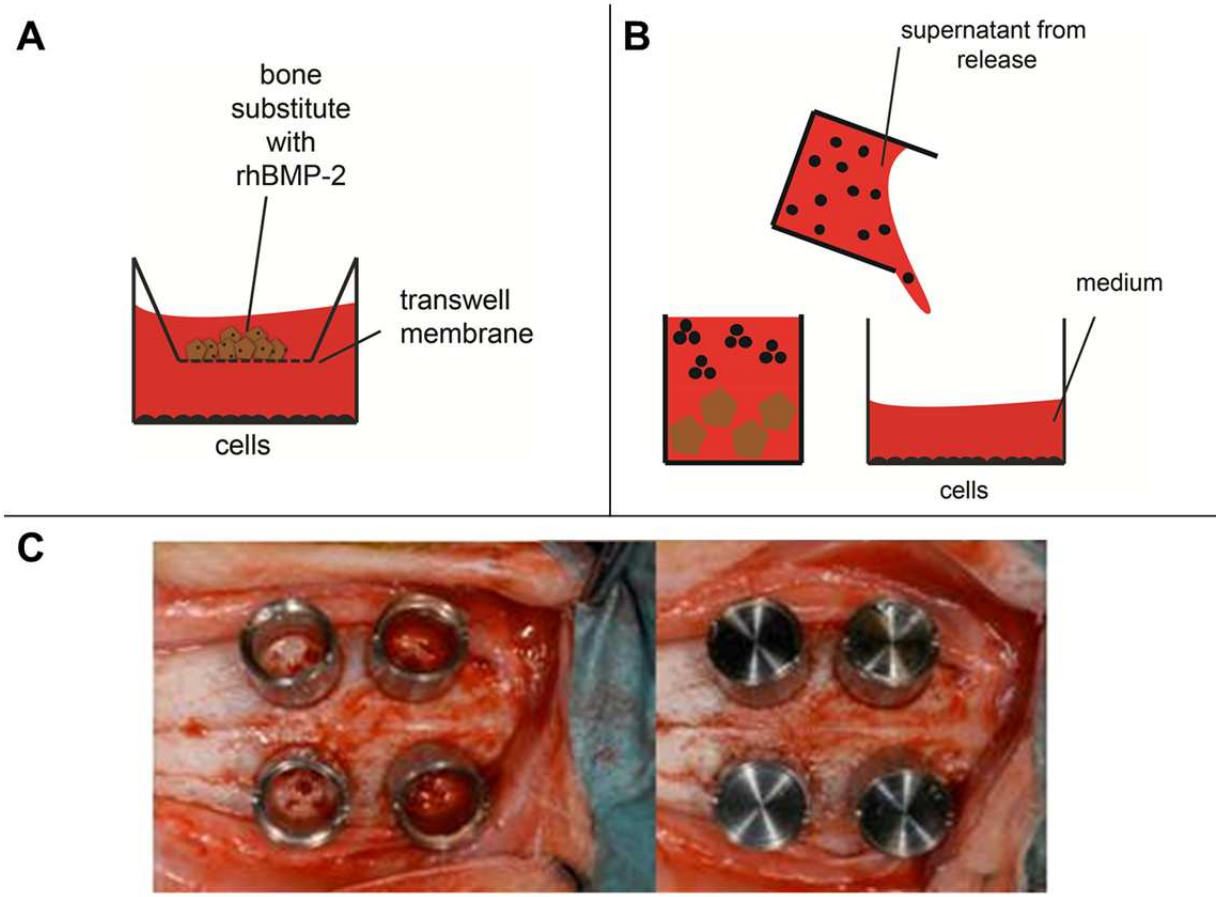

Figure 1). Samples and controls were prepared as described in section 2.1.4. Concentration of ^{125}I-rhBMP-2 adsorbed to the apatite granules was determined by measuring radioactivity of the whole sample and the supernatant with a Cobra II gamma counter. The liquid remaining from the adsorption procedure was discarded subsequently and the rhBMP-2 loaded materials were transferred to new tubes containing differentiation medium (MEM-α (Invitrogen, CA, USA) supplemented with 10% FBS (Invitrogen,), ascorbic acid (50 mg/l, SigmaAldrich, MO, USA) and antibiotics (100 U/ml penicillin G and 100 mg/ml streptomycin, Invitrogen)). The final volume was calculated to be 1 ml, the volume of apatite granules not taken into account. The amount of ^{125}I-rhBMP-2 transferred to the new tubes was determined by measuring the radioactivity of the whole samples. After incubation of the controls for 1h, allowing interaction of the bone substitute materials with the differentiation medium, 1.9 µg ^{125}I-rhBMP-2 were added. All samples were incubated at 37 °C. The amount of ^{125}I-rhBMP-2 in the whole samples as well as in 10 µl of the supernatant was evaluated at various time points by measuring the gamma radiation. The amount of ^{125}I-rhBMP-2 adsorbed to apatite granules or in solution was evaluated by comparing values from the samples with the

positive controls. The removed supernatant was not replaced but taken into account for calculations of the cumulative release/adsorption. Each sample and control was prepared six times (n=6) and the experiment was repeated. After 15 days of incubation, the supernatant of the samples was evaluated on SDS-PAGE gels and using FPLC.

2.2. Cell Experiments

2.2.1 Cell Culture Maintenance

MC3T3-E1 cells (Subclone 24, ATCC, VA, USA) were expanded in proliferation medium (MEM-α (w/o ascorbic acid) (Invitrogen) supplemented with 10% FBS (Invitrogen) and were used for experiments between passage three and ten. Cells were never allowed to become confluent and split in a ratio of 1:5. All experiments were carried out in differentiation medium. For experiments in 24- or 48-well plates, cells were seeded at a density of 10'000 cells/cm^2.

2.2.2 Assay for Alkaline Phosphatase Activity

Cells were stimulated for 7 days at 37 °C and 5% CO_2. They were subsequently lysed using 0.56 M 2-amino-2-methyl-1-propanol (SigmaAldrich) pH 10 containing 0.2% Triton X-100 (SigmaAldrich). Alkaline phosphatase (ALP) activity in the lysis buffer was determined via the kinetics of a colour reaction based on the reduction of p-nitrophenyl phosphate (SigmaAldrich) to p-nitrophenol. The DNA concentration of the lysate was determined using a PicoGreen assay according to the instructions of the manufacturer (Invitrogen). Optical density of the ALP activity reaction product p-nitrophenol (410 nm) and fluorescence of DNA bound PicoGreen (ex/em ~480 nm/~520 nm) was detected with a Synergy HT plate reader (Biotek, VT, USA). The determined ALP activity was adjusted to the total cell number by standardizing it to the DNA concentration in the sample.

2.2.3 Cell Stimulation with completely released rhBMP-2

rhBMP-2 was released into differentiation medium from DBBM and HA/TCP granules for 15 days as described in section 2.1.4 and section 2.1.5. The conditioned differentiation medium supernatant from samples and controls was diluted 1:1 in fresh differentiation medium before stimulating MC3T3-E1 cells. Cells were incubated at 37 °C and 5% CO_2, and osteoblastic differentiation was evaluated after 7 days of incubation by an ALP activity assay as described earlier.

2.2.4 Continuous Stimulation of Cells with Transwell Membranes

DBBM and HA/TCP granules loaded with 1 µg rhBMP-2 as well as controls were prepared as described in section 2.1.4. MC3T3-E1 cells were seeded in 1 ml cell culture medium on the bottom of 24-well plates in differentiation medium. rhBMP-2-loaded granules and control granules were placed in the wells containing the cells, separated from the cells via a transwell membrane insert (Polyester (PET) Membrane Transwell-Clear Inserts, 24-well plate, 3.0 µm pore size, Corning, NY, USA) permeable for rhBMP-2. rhBMP-2 for positive controls was added to the upper part of the transwell compartment and cells were incubated at 37 °C, 5% CO_2. Osteoblastic differentiation was evaluated by an ALP activity assay as described in section 2.2.2 after 7 days of incubation.

2.3. Animal Experiments

2.3.1 Cleaning and Sterilization of Titanium Cylinders

Titanium cylinders were manufactured in the machine shop at the Materials Department of ETH Zurich (Switzerland) as described earlier [22]. Cylinders were immersed in approximately 50 ml acetone, sonicated for 15 min and rinsed with plenty of water (3x), immersed in approximately 50 ml 2-propanol, sonicated for 15 min and rinsed with plenty of water (3x), immersed in approximately 50 ml of ultrapure water (MilliQ, Millipore, MA, USA) dried at 37 °C overnight and vapor sterilized (134 °C) in the central sterilization facilities of the University Hospital Zurich (Switzerland).

2.3.2 Functionalization of Apatite Granules with rhBMP-2

DBBM and HA/TCP were plasma cleaned in a radio-frequency oxygen plasma chamber (RF plasma chamber) at an oxygen pressure of 0.1 mbar. Weight per volume of granules was determined for both DBBM and HA/TCP by filling a 0.2 ml RT/PCR tube and determining its weight increase. Based on these results, 750 mg of DBBM or 825 mg HA/TCP were mixed with 3000 µl 1 mM HCl containing 75 µg rhBMP-2. By choosing these ratios, the amount of bone substitute needed to fill a cylinder was loaded with 10 µg rhBMP-2 for both materials. The mixture was vortexed, briefly spun down (10600xg, 10s) in a centrifuge and incubated overnight. Controls were prepared without the addition of rhBMP-2. The next day the samples were frozen by immersion in liquid nitrogen and lyophilized.

2.3.3 Surgery

6 rabbits were sedated by Ketamin and further anaesthetized by an Isofluran-N_2O inhalation method. A linear incision was made from the nasal bone to the midsagital crest. After the

deflection of the soft tissues, a sub periosteal dissection of the operation site was performed (occipital, frontal, and parietal bones). To place the specially designed cylinder made of c.p. titanium with a machined surface four evenly distributed 6 mm diameter circular slits with a 1mm sink depth were created and perforations of the external cortical plate inside the slits were prepared. The cylinders measured 7 mm in height and 7 mm in outer diameter and exhibited a screw design toward the bone site and a small shoulder for a titanium lid toward the covering skin flap. The surgical area was rinsed with saline to remove bone debris and four cylinders were screwed in the prepared slits, providing primary stability. Next, the cylinders were filled with DBBM, DBBM/rhBMP-2, HAT/TCP, or HA/TCP/rhBMP-2. The cylinders fillings were assigned in a random systematic manner. The cylinders were left open toward the bone but were closed with a titanium lid designed to press fit into the opening. After placement of the lids, the soft tissues were closed with sutures. Analgesia was provided by injection of Novalgin® (50 mg/kg; Aventis Pharma AG, Zürich, Switzerland). At 8 weeks the rabbits were sacrificed after sedation with barbiturates by an overdose of Ketamin® (Pfizer AG, Zürich, Switzerland) and the calvarial bone was excised.

2.3.4 Histological analyses

Samples were prepared as described earlier [23]. In brief, the samples were first prepared with a sequential water substitution process and further infiltrated by methyl methacrylate. Samples were allowed to polymerize at 37°C in an air tight water bath. The specimens were sectioned in the frontal plane through the middle of the cylinders. Sections of 200 μm thickness were obtained, ground, and polished to a uniform thickness of 60–80 μm. The specimens were surface stained with toluidine blue.

2.3.5 Histomorphometry

Measurements were carried out on a picture mosaic taken from the entire area of interest via a light microscope at a magnification of 160x, using a superimposed test grid of points and cycloid lines. The numbers of test points overlying the profiles of the different components (i.e. mineralized bone tissue, non-mineralized tissue and graft particles) were counted. Test points are defined and symbolized according to the standard nomenclature of the International Society for Stereology [24]. The graft to bone contact was calculated by the number of intersections between graft particles and the outlines of either mineralized bone or non-mineralized tissue.

In addition, a quantitative evaluation of the area of bone augmentation within the cylinders was carried out to extract a clinically relevant parameter. First digital images were obtained and processed with an image analysis program (Adobe Photoshop CS3). The pixel counts

were directly carried out on the digital images and comprised the area of newly formed bone, including bone marrow and osseointegrated bone substitute particles.

2.4. Statistical Analysis

The primary unit of analysis was the animal. Mean values and standard deviations were calculated for the area of osseous regeneration, absolute bone points, absolute bone substitute points, and for the percentage of bone-to-bone substitute contact in the middle section within the cylinder. The significance of differences was evaluated by the Friedman test followed by Wilcoxon signed rank test. The limit for significance was set to <0.05. Statistical analysis was performed by using a statistical software package (IBM SPSS Statistics Version 21 for Windows).

3. Results

3.1. Characterization of the Iodinated rhBMP-2

rhBMP-2 was labeled with radioactive iodine (^{125}I-rhBMP-2) in order to simplify the detection of the released growth factor. We evaluated the functionalized protein by means of SDS PAGE and FPLC. The FPLC chromatograms of rhBMP-2 and ^{125}I-rhBMP-2 are alike (Figure 2 B) and we detected similar MW bands for ^{125}I-rhBMP-2 and rhBMP-2 on reduced SDS PAGE gels. However, we observed a second band for ^{125}I-rhBMP-2 on the non-reduced SDS-PAGE gels (Figure 2 A; lane 2 and 5).

3.2. Release Profiles from Substitute Materials

Overnight incubation of rhBMP-2 to the apatite materials in 1 mM HCl led to the adsorption of 67% ± 5% and 51% ± 7% of the applied ^{125}I-rhBMP-2 to HA/TCP and DBBM respectively. To study the release of the adsorbed rhBMP-2, the apatite granules were transferred to new tubes containing differentiation medium. The release profile of previously adsorbed ^{125}I-rhBMP-2 from HA/TCP and DBBM samples (Fig. **Fehler! Verweisquelle konnte nicht gefunden werden.**3A) as well as the interaction of ^{125}I-rhBMP-2 with HA/TCP and DBBM controls in differentiation medium (Fig.**Fehler! Verweisquelle konnte nicht gefunden werden.** 3B) was evaluated by determining concentrations of the growth factor in the supernatant and in the whole sample using a gamma counter. After an initial burst release, DBBM showed a slow release during 15 days and was capable of retaining half of the

adsorbed growth factor. ^{125}I-rhBMP-2 showed a strong affinity to DBBM in the controls, even if the DBBM was preincubated in FBS prior to its immersion in differentiation medium. HA/TCP samples showed a burst release and the complete amount of adsorbed ^{125}I-rhBMP-2 was released after 24h. We did not observe any interactions between ^{125}I-rhBMP-2 in the controls.

3.3. Biological Activity of Released rhBMP-2

Effect of rhBMP-2 released from DBBM and HA/TCP on MC3T3-E1 was evaluated *in vitro* by stimulating them either with the supernatant of a 15 days release (endpoint, section 2.1.3) or by separating the cells from the rhBMP-2 loaded granules via a transwell membrane permeable for rhBMP-2 (continuous, section 2.3.40). Cell viability was affected by the presence of DBBM in the continuous approach as indicated by a reduced cell number (Figure 4 A). The presence of rhBMP-2 slightly increased cell viability in all samples.

We did not detect any significant differences between ALP activities of cells stimulated by the endpoint or the continuous method (Figure 4 B). While functionalization of HA/TCP with rhBMP-2 leads to an ALP response comparable to the positive controls containing the same amount of rhBMP-2 but no apatite granules, the combination of DBBM and rhBMP-2 leads to a reduced ALP activity compared to the positive control.

3.4. *In vivo* bone augmentation model

After operation, all animals showed uneventful healing of the area of surgery. In none of the samples signs for immunoreactions or inflammation were seen. The external and internal cortical plates of the cranium were clearly visible on the histological specimens (Figure 5). The toluidine blue staining allowed a clear distinction between the graft material and the regenerated bone (Figure 5). Bone formation close to the skull was more frequent, indicating an upwards movement of the bone front, even in the rhBMP-2 loaded samples.

The area of bone formation comprises the area where bone formation had occurred; specifically the area where ingrowing interconnected bone tissue has covered bone substitute materials. Compared to all other groups the values for the DBBM/rhBMP-2 group was significantly higher (Figure 6 A), indicating that in the presence of rhBMP-2 in combination with DBBM bone augmentation was enhanced. The number of bone points which equals new bone formation, however, did not vary significantly between the groups (Figure 6 B). Therefore rhBMP-2 accelerated mainly the advancement of the bone front from bottom to the top of the cylinder in the DBBM/rhBMP-2 group.

Although both HA/TCP and DBBM are apatites, their geometry, microporosity, and the degradation in particular of the TCP vary (Table 1), resulting in different apparent densities of the granules that result in a higher number of bone substitute material points in the DBBM/rhBMP-2 group compared to both HA/TCP containing groups (Figure 6 C).

Of clinical relevance is the extent of the area of bone formation (Figure 6 A) but also the integration of the bone substitute into newly forming bone, since it is an indication for the stability of the resulting bone/bone substitute construct and indicative for its integration into normal bone turn over. The level of bone to bone substitute material interface is 36.0 ± 2.9% for DBBM and 33.7 ± 3.8% for DBBM/rhBMP-2 respectively. For HA/TCP, however, this number was much lower with 24.0 ± 3.5% but was significantly increased by BMP to 33.2 ± 1.4% in the HA/TCP/rhBMP-2 group (Figure 6 D).

4. Discussion

We here evaluated two clinically applied apatite based bone substitute materials in terms of their potential for a combination with the osteoinductive growth factor rhBMP-2. In a previous study we used an ELISA specific for rhBMP-2 to detect the growth factor [21]. However, this method has several drawbacks. The growth factor is recognized by an antibody and its detection is therefore dependent on the accessibility of the recognized epitope. Although the epitope recognition could be indicative for the proper folding and bioactivity, this assay is very susceptible to buffer changes and the presence of other molecules. We therefore labeled rhBMP-2 with radioactive iodine (^{125}I) via the Iodogen method. Evaluating the labeled growth factor by comparing its FPLC size exclusion chromatogram to the native rhBMP-2 we did not find any differences (Figure 2 B). We further compared native rhBMP-2 and ^{125}I-rhBMP-2 on an SDS PAGE gel under reducing and non-reducing conditions. While we did not find any differences between the molecular weight detected in case of the reduced samples, we found a weak additional band in case of ^{125}I-rhBMP-2 under non-reducing conditions (Figure 2 A) indicating that some of the dimers might have undergone a slight modification in their secondary structure which is reflected in a reduced bioactivity of the growth factor (data not shown). The data suggests that the primary structure of the growth factor was not affected by the iodination and the secondary structure was only affected in a small portion of the applied rhBMP-2.

In a clinical setting the granules can be loaded in the operation theatre and then transferred into the patient. Therefore we studied the release from loaded granules to mimic better clinical conditions. In our previous study [21] we added the cell culture medium to the same tubes where the adsorption of rhBMP-2 from 1 mM HCl was performed. Determining the

amount of rhBMP-2, we found that more rhBMP-2 was transferred by HA/TCP than had been transferred by DBBM. The release profile of rhBMP-2 from DBBM however confirmed our previous results showing a high affinity of rhBMP-2 to DBBM. DBBM was capable of retaining half of the adsorbed growth factor in differentiation medium during 15 days (Figure 3 A). In contrast, all rhBMP-2 adsorbed to HA/TCP was released during the initial 24h. These results are confirmed by the control samples where the apatite materials were preincubated in differentiation medium for 1h and the rhBMP-2 was added subsequently. We further preincubated DBBM in FBS overnight and still saw adsorption of rhBMP-2. Therefore, rhBMP-2 adsorbs to DBBM even in the presence of serum proteins [21]. In contrast to this there was no interaction between rhBMP-2 and HA/TCP in the control samples (Figure 3 B), indicating that the growth factor has a low affinity to HA/TCP under physiological conditions and that the investigated HA/TCP granules are not a slow release system for rhBMP-2. This lack of affinity of rhBMP-2 for HA/TCP could be the reason for the failure to get AMPLIFY™ cleared for use in humans, since the high load of rhBMP-2 might have been release too fast, was not retained at the application site and induced cancer formation at various locations in the body [5].

DBBM is mainly composed of hydroxyapatite. The difference in absorption and release of rhBMP-2 might not derive from the difference between hydroxyapatite and β-tricalcium phosphate, since it was shown by others that after 24 h 49.6% of rhBMP-2 can still be associated with β-tricalcium phosphate and 34.0 % with hydroxyapatite [25]. In that study the specific surface of both materials was very similar ranging from 1 to 4 m^2/g. In our case, however, DBBM has a surface area of 67 m^2/g and the HA/TCP of 0.009 m^2/g. This suggests that the affinity and release kinetic of rhBMP-2 to calcium phosphates is determined mainly by the specific surface area, which is extremely high with DBBM (Table 1). This conclusion is in line with results obtained for other proteins [20].

Stimulation of MC3T3 cells with rhBMP-2 released from both HA/TCP and DBBM revealed that most of the released growth factor was bioactive. Since there was more rhBMP-2 transferred by HA/TCP than by DBBM and half of the transferred rhBMP-2 was retained by DBBM, there was much more ALP activity induced in samples with HA/TCP than those with DBBM (Table 1). We did not observe a marked difference in ALP activity between cells stimulated with the supernatant from a release (endpoint) or continuously stimulated by rhBMP-2 released from the bone substitute materials separated from the cells via a transwell membrane permeable for rhBMP-2. This suggests that after the burst released rhBMP-2, the rhBMP-2 that was slowly released from DBBM between day 2 and day 7 was either not bioactive or its amount too low, or the *in vitro* release kinetic ill-suited to be detected using this assay. This is in agreement with our previous results that did not show any slow release

using an ELISA for the detection of rhBMP-2 which would not detect growth factors with a compromised structure [21].

Most *in vitro* tissue culture experiments represent a closed compartment where factors cannot diffuse out. In the clinical situation a closed compartment can be generated by the application of a membrane, a technique called guided bone regeneration, which is applied frequently in dentistry to augment sufficient bone for the placement of dental implants. To mimic this situation and to test both materials for bone augmentation, which is very demanding in the clinical setting, we applied our cylinder model, where titanium cylinders are placed on top of the calvarial bone and the content is restricted by the calvarial bone on one side and by titanium on all other sides [23] (Fig 1C). Bone augmentation, determined by the area of osseous regeneration, was significantly higher in the DBBM/rhBMP-2 group compared to all other groups. Therefore the efficiency of DBBM for bone augmentation could be further enhanced by the combination with rhBMP-2. Since the majority of the rhBMP-2 is bound to the DBBM granules it also suggests that even the rhBMP-2 strongly bound to DBBM retains sufficient bioactivity to push the bone augmentation front further. That tightly bound physio-absorbed rhBMP-2 can still be bioactive was shown for other materials before [26].

The fast released rhBMP-2 in the HA/TCP/rhBMP-2 group estimated to be double the amount released from DBBM, however, failed to increase bone augmentation significantly. *In vitro*, all samples exposed continuously to DBBM showed a decrease in proliferation and ALP activity, which might derive not only from the high affinity of DBBM for rhBMP-2 but also for other proteinaceous factors, depriving the culture medium of factors needed by the cells in this artificial environment. *In vivo*, however, overall protein content is much higher. This effect, which most likely is due to the 1000 times higher specific surface area of DBBM compared to HA/TCP (Table 1) might not play any negative role at all. Therefore *in vitro* cell culture based tests of biomaterials have to be complemented by *in vivo* models.

Recently it was shown that especially designed fast degrading TCP in contrast to slow degrading hydroxyapatite based materials are osteoinductive due to a release kinetic of calcium ions which can trigger signaling pathways via the protein kinase C pathway, leading to an increase in the expression of BMPs [8, 27]. For the ceramics we used in this study, neither the *in vitro* nor the *in vivo* results indicate any osteoinductive potential. Therefore the marked difference in the specific surface area appears to be the main reason for the superior performance of the DBBM *in vivo*. This is further supported by findings that an increase in specific surface area enhances not only the adsorption of proteins from plasma including fibronectin and vitronectin, but at the same time also stimulates osteoblast adhesion, growth and osteogenic differentiation [20].

In a clinical trial to study bone augmentation for the placement of dental implants with DBBM with and without rhBMP-2 the amount of newly formed bone was not increased significantly but bone maturation and bone to DBBM contact was [28, 29]. Here the space was constrained by a guided bone regeneration membrane serving as a barrier to enhance bone regeneration. Although the time between operation and evaluation in the clinical trial was 6 months the combination of DBBM with rhBMP-2 improved its performance significantly and supports our findings that rhBMP-2 bound to DBBM is bioactive and still capable to enhance bone augmentation. Therefore this combination appears very safe, since it induces bone formation just at the site where the material was placed.

Bone to bone substitute contact was high for DBBM and could not be increased further when rhBMP-2 was applied. For HA/TCP, however, the initial level of bone to bone substitute contact was low and increased by the addition of rhBMP-2. Our *in vitro* experiments show that rhBMP-2 is not adsorbed by HA/TCP granules. This increase in bone to HA/TCP contact therefore indicates that the burst released rhBMP-2 still had some significantly positive effects in combination with HA/TCP, in particular since the diffusion was restricted by the titanium cylinder mimicking a guided bone regeneration scenario. The positive effect could further be caused by the lyophilization procedure during the preparation of the samples. Small amounts of rhBMP-2 clusters could remain attached to the surface and induce a better bone/bone substitute interface.

5. Conclusions

The comparison of two ceramics as potential delivery systems for rhBMP-2 revealed that DBBM, which has a 1000 fold higher specific surface area, adsorbs rhBMP-2 efficiently and keeps 50% of it attached for more than two weeks. From the HA/TCP material all rhBMP-2 is released in a burst during the first 24h. The released rhBMP-2 from both materials remains bioactive. *In vivo* DBBM performs better in terms of bone augmentation and bone to implant contact. By the addition of rhBMP-2 both parameters are further improved and bone augmentation is significantly higher. In combination with rhBMP-2 both parameters are increased for HA/TCP but only bone to bone substitute contact increased significantly. Therefore the addition of rhBMP-2 can cope to some extend for a low specific surface area. DBBM appears to be a very safe delivery system for rhBMP-2 since 50% of the rhBMP-2 remains absorbed but active, keeping the rhBMP-2 activity restricted to the site where the osteoinductive bone substitute was placed.

6. Acknowledgement

The authors want to thank Yvonne Bloemhard and Alexandr Tchouboukov and Susan Cohrs for their technical assistance.

7. References

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8. Figure legends

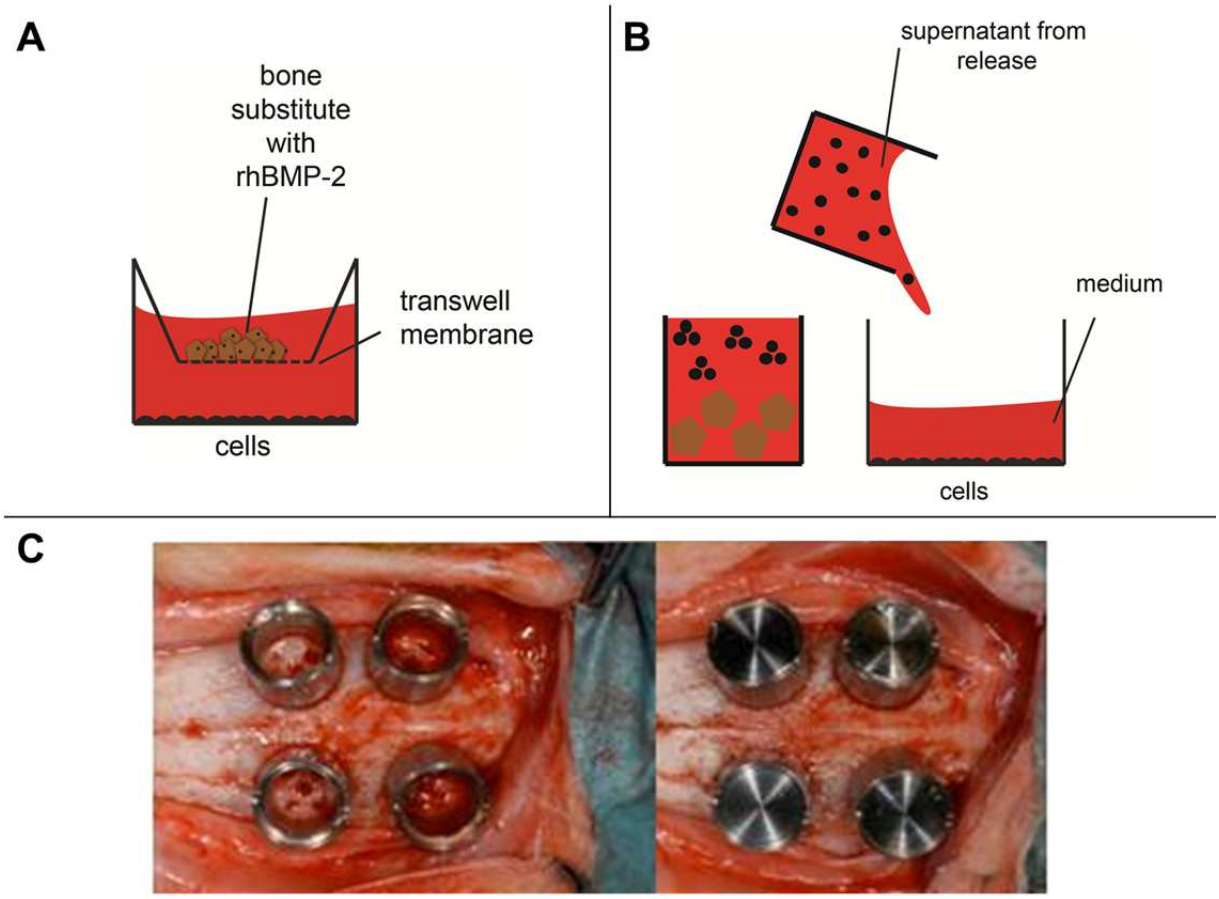

Figure 1: *In vitro* and *in vivo* settings. A) Schematic drawing of the setup for in vitro release. B) Schematic drawing of the setup for cell culture experiments. Endpoint stimulation: rhBMP-2 was released from granules into differentiation medium for 15 days. Granules were separated from the supernatant that was used to stimulate MC3T3-E1 cells. Continuous stimulation: rhBMP-2 loaded granules were separated from cells via a transwell membrane permeable for rhBMP-2. C) 4 titanium cylinders are placed on top of the calvarial bone (left panel). After filling with materials, the cylinders are closed by a press fitted titanium lid (right panel).

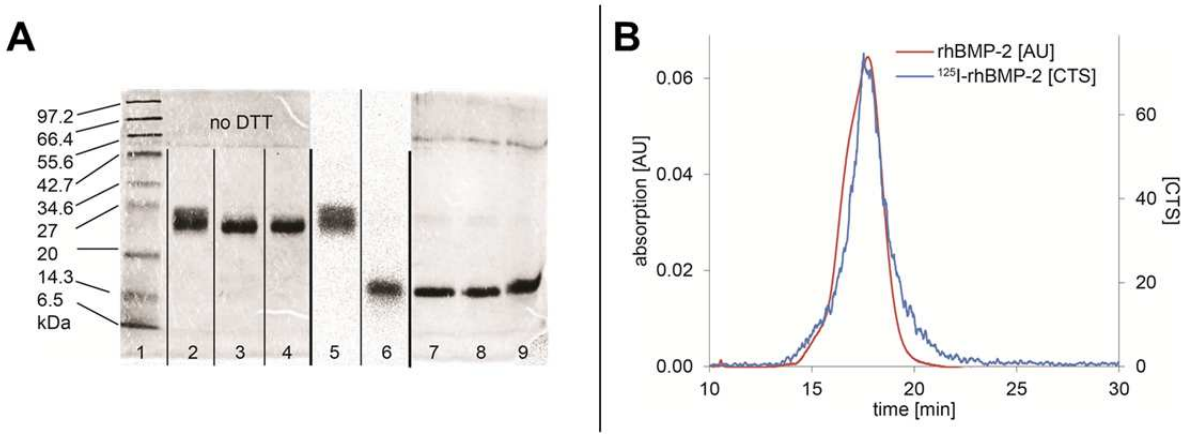

Figure 2: Evaluation of the iodination of rhBMP-2. A) SDS PAGE gel stained with Coomassie and investigated for autoradiography. 1) marker; 2) 1 µg ^{125}I-rhBMP-2; 3) & 4) 1 µg rhBMP-2; 5) & 6) 80 ng ^{125}I-rhBMP-2; 7) & 8) 1 µg rhBMP-2; 9) 1 µg ^{125}I-rhBMP-2. Bands of lanes 5) and 6) were detected by autoradiography. While there is no difference in MW between rhBMP-2 and ^{125}I-rhBMP-2 if the protein has been reduced by DTT, a second band can be observed for ^{125}I-rhBMP-2 under non-reducing conditions (lane 2 and 5). B) Typical FPLC chromatogram of rhBMP-2 and ^{125}I-rhBMP-2 detected via UV detector and scintillation counter respectively. We did not observe differences between the chromatograms of the labeled and the unlabeled protein.

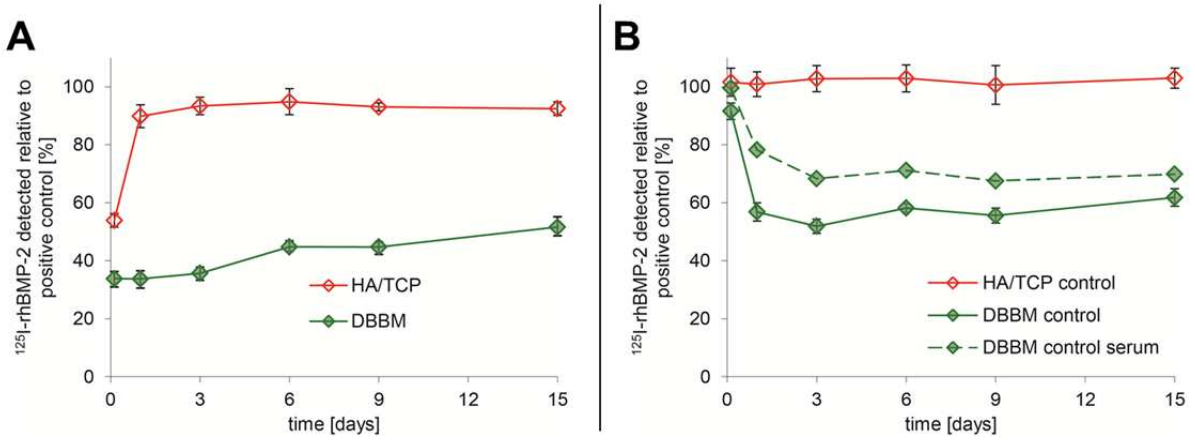

Figure 3**:** ^{125}I-rhBMP-2 release and absorption from and to HA/TCP and DBBM. A) *In vitro* release of ^{125}I-rhBMP-2 from HA/TCP and DBBM into differentiation medium at 37 °C. While almost all ^{125}I rhBMP-2 is immediately released from HA/TCP, DBBM is capable of retaining a large fraction of ^{125}I-rhBMP-2 during 15 days of incubation. B) *In vitro* absorption of ^{125}I-rhBMP-2 to HA/TCP and at 37 °C. While there is no interaction between ^{125}I-rhBMP-2 and HA/TCP granules in cell culture medium, the growth factor adsorbs to DBBM even in the presence of FBS in the cell culture medium. Preincubation of DBBM with FBS does not prevent ^{125}I-rhBMP-2 from adsorbing, indicating that the affinity of ^{125}I-rhBMP-2 to DBBM is higher than the affinity of serum proteins.

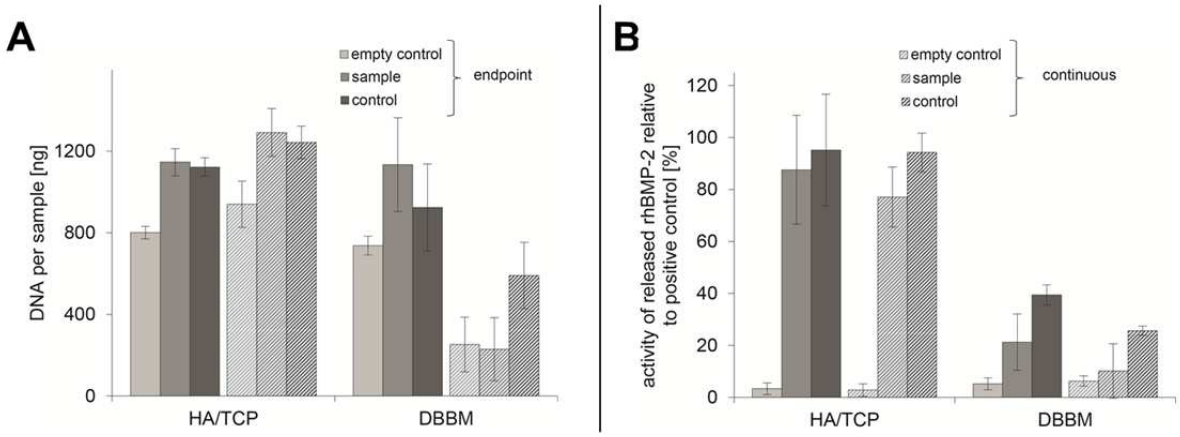

Figure 4**:** Biological activity of rhBMP-2 released from DBBM and HA/TCP. A) Cell viability by DNA content. rhBMP-2 increases cell viability. The presence of DBBM *in vitro* (continuous method) affects cell viability. B) ALP activity induced by the released rhBMP-2. While almost all rhBMP-2 is released from HA/TCP that induces an ALP activity only slightly lower than in the positive controls, large amounts of rhBMP-2 are retained by the DBBM resulting in a much lower ALP activity.

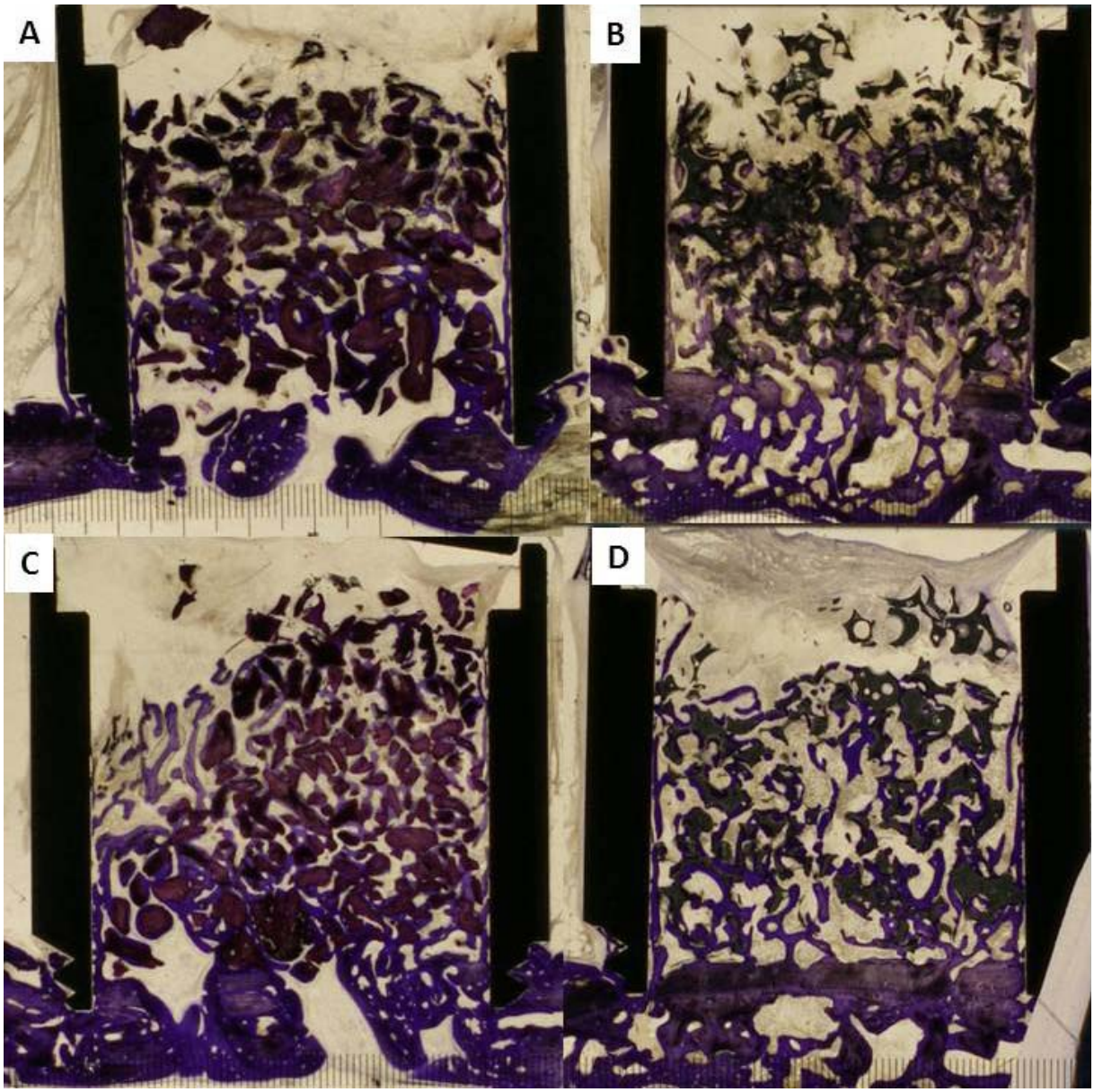

Figure 5: Bone augmentation by bone substitutes. In the rabbit cylinder model, bone augmentation was compared between cylinders filled with DBBM (A), HA/TCP (B), DBBM/rhBMP-2 (C) and HA/TCP/rhBMP-2. Bone is stained by toluidine blue as bright blue, bone substitutes are stained purple (DBBM) or black (HA/TCP). The titanium cylinders to the left and right side of each sample appears black. In the lower part of each sample the original calvarial bone is seen. The scale provided in the lower part shows 0.1 mm.

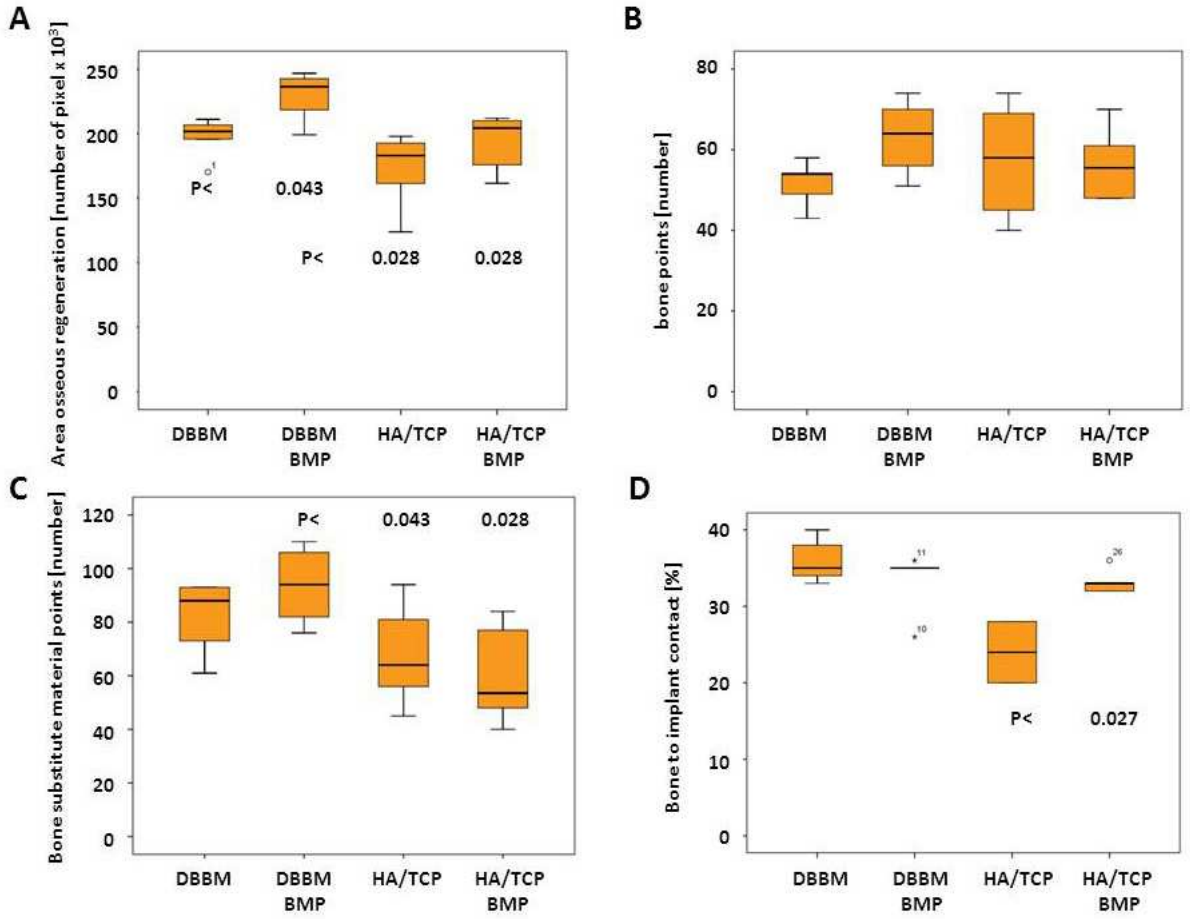

Figure 6: Histomorphometric results from the bone augmentation experiment. For all four materials, DBBM, DBBM/rhBMP-2, HA/TCP and HA/TCP/rhBMP-2, the area of osseous regeneration (A), absolute bone points (B), absolute bone substitute points (C), and bone to implant contact (D) is displayed. Values are given as box-plots ranging from the 25th (lower quartile) to the 75th (upper quartile) percentile including the mean and whiskers showing the minimum and maximum values. The P values of all statistically significant differences are provided.